

TREATMENT OF TRAUMATIC BRAIN INJURY USING METHYLENE BLUE
INFUSION

Undergraduate Honors Thesis

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Abstract:

Traumatic brain injury (TBI) is a significant epidemic worldwide. Our lab has shown that methylene blue (MB), a known anti-inflammatory compound, reduces acute inflammatory and behavioral deficits after TBI. Nevertheless, a single dose of MB was unable to significantly improve functional recovery following TBI. Here, we examined the neuroprotective effects of multiple doses of MB on functional recovery up to 7 days after TBI and LPS-mediated immune challenge one month after TBI. Adult BALB/c mice (2-3 mo) received a moderate, diffuse midline fluid percussion injury and were administered intravenous MB 15 min, 12 h, and 24 h after injury. MB treatment improved motor coordination one week after TBI. Furthermore, our data indicate that MB reduces inflammatory gene expression (CCL2, CD14, IL-1 β , TNF α) and attenuates markers of glial reactivity (MHCII) following an LPS challenge one month after injury. Additionally, MB improves cognition one month after injury. Collectively, the results demonstrate that multiple doses of MB may be a suitable treatment for acute TBI-related deficits and help prevent complications due to secondary immune challenges.

Introduction:

Traumatic brain injury (TBI) is a leading cause of death worldwide in individuals under the age of 45 [1]. According to the CDC, over 2.5 million TBIs are sustained each year in the United States. This is, in part, because while individuals may recover from the immediate consequences of TBI (e.g., dizziness, motor dysfunction, and confusion), long-lasting neuropsychiatric complications and cognitive decline develop and persist. Therefore, TBI-related disabilities often have permanent, profound effects on everyday life. As interventions to prevent these issues from developing are unavailable, TBI is one of our largest health concerns.

Mild to moderate TBIs are the most prevalent and are associated with motor and neurological complications and disabilities. A traumatic brain injury has two parts: (1) primary damage which occurs at the time of injury and (2) secondary damage incurred by delayed inflammatory responses. Primary damage represents the direct biomechanical damage inflicted by the injury and can only be influenced by preventive measures [2]. This includes disruption of neuronal and glial cell membranes, damage to vascular structures including the blood brain barrier (BBB), tissue loss or cell death, and diffuse axonal injury [3]. Secondary damage is sensitive to therapeutic treatment and is characterized by delayed physiological, cellular, and molecular responses intended to restore homeostasis [4]. Therefore, treatment strategies that target the initial injury may minimize secondary damage and improve overall prognosis.

Mild and moderate TBIs, those that are most sustained and associated with motor and neurological complications and disabilities, are best represented by a moderate/diffuse TBI model, such as midline fluid-percussion injury (FPI). The release of prostaglandins, free radicals, complement factors, pro-inflammatory cytokines, and other inflammatory mediators can increase neuronal death and tissue loss beyond that of the primary injury. Moreover, this increase in brain

inflammation may initiate a cascade of biochemical events that lead to the development of depression [5]. In one study, 42% of 722 TBI patients were found to suffer from major depressive disorder an average of 2.5 years after injury [6]. Currently, treatment for TBI is directed towards treating TBI-associated complications as they arise, including edema and hypotension. Unfortunately, there are no effective pro-active treatment strategies to alleviate the inflammatory response or prevent neuropsychiatric complications.

Microglia, the innate immune cells of the central nervous system (CNS), are responsible for mediating acute inflammatory processes in the brain. Microglia are activated in response to immune challenge in the periphery (e.g., viral infection) or by psychological stress and CNS injury. Microglial activation is determined by pro- and anti-inflammatory cytokine expression or by morphological change, in which cells demonstrate an active phenotype characterized by shortened and thickened processes and enlarged cell bodies [10]. Microglial activation helps alter physiological and behavioral responses in order to fight infection, avoid threatening situations, or repair the CNS. Prolonged activation, which can occur as a result of microglial priming, can lead to the development of long-lasting behavioral dysfunction. Microglial priming, otherwise defined as hypersensitization, can be visualized by mRNA MHCII expression and stems from a variety of factors, including infection, stress, and age. Primed microglia are hyper-reactive in response to peripheral immune challenge, causing behavioral deficits.

Our lab has characterized microglial priming in models of aging, repeated social defeat, early life infection, and TBI in mice. Microglial priming is important because after a secondary challenge, primed microglia become hyper-inflammatory, resulting in exaggerated neuroinflammation and the development of behavioral deficits. For example, as a result of normal aging, microglia have increased expression of MHCII [11,12]. Increased expression of

MHCII is also observed after TBI model at 24 h. Lipopolysaccharide (LPS) is derived from the cell surface of gram-negative bacteria and is used to induce a sterile immune challenge. Following LPS challenge, primed microglia secrete increased levels of the pro-inflammatory cytokine IL-1 β [13,14], resulting in prolonged sickness response [11] and depressive-like behavior [12] in aged mice. Three days after TBI, the acute inflammatory response resolves, and behavioral deficits resolve within 7 days. More importantly, long after injury (30 d), microglia maintain a primed and pro-inflammatory phenotype. Furthermore, one month following TBI, microglia have increased MHCII expression and, following a peripheral immune challenge with LPS, these primed microglia are hyperactive and produce exaggerated levels of pro-inflammatory cytokines [15]. In addition, these mice had prolonged sickness behavior and developed depressive-like behavior corresponding with exaggerated neuroinflammation [15]. Thus, the presence of primed microglia represents a state of CNS dysfunction; they are sensitized and highly reactive to subsequent inflammatory challenges.

Methylene blue (MB), an antioxidant, inhibits inflammation by diverting electron flow from radical-forming oxidases [7]. MB is used clinically to reduce inflammation associated with sepsis that results from the production of reactive oxygen species by enzymes including inducible nitric oxide synthase (iNOS) [8,9]. In a model of focal TBI, MB treatment minimized lesion volume, behavioral deficits, and neuronal degeneration as observed up to 14 d after injury [16]. Thus suggesting the use of methylene blue in other models of TBI.

After observing that TBI leads to behavioral complications, we wanted to identify a clinically-relevant pro-active treatment that would reduce microglial activation and prevent TBI-associated complications. Through collaborating with Dr. Daniel Eiferman, a clinician in the Department of Surgery at the OSU Medical Center, we began to investigate MB as a potential

therapeutic. MB is safe, readily crosses the blood-brain barrier, and could be administered intravenously within minutes after a head-injury. We have recently published that immediate intervention with MB is effective in improving short-term complication up to one week after a TBI [17]. Cell culture experiments with LPS-activated BV2 microglia confirm that MB treatment directly reduces IL-1 β and increases IL-10 messenger ribonucleic acid in microglia. MB intervention attenuates TBI-induced inflammatory gene expression (IL-1 β , TNF α) in enriched microglia/macrophages 1 d post injury, suggesting that MB attenuates neuroinflammation by reprogramming key inflammatory mediators. MB reduces cerebral edema, microglial activation, and neuroinflammation 24 h after a TBI. More importantly, these MB-induced reductions in brain edema and inflammation correspond to a reduction in TBI-associated depressive-like behavior up to seven days after a TBI [17]. There were no improvements in other aspects of functional recovery, however, including anxiety, motor coordination deficits, and nesting behavior and long-term effects were insignificant, indicating that our treatment strategy could be improved. We hypothesized that multiple doses of methylene blue administered immediately after a traumatic brain injury reduces microglial activation and priming following an immune challenge at one month after injury as well as result in the improvement of motor coordination at one week and cognition at one month after injury (figure 1).

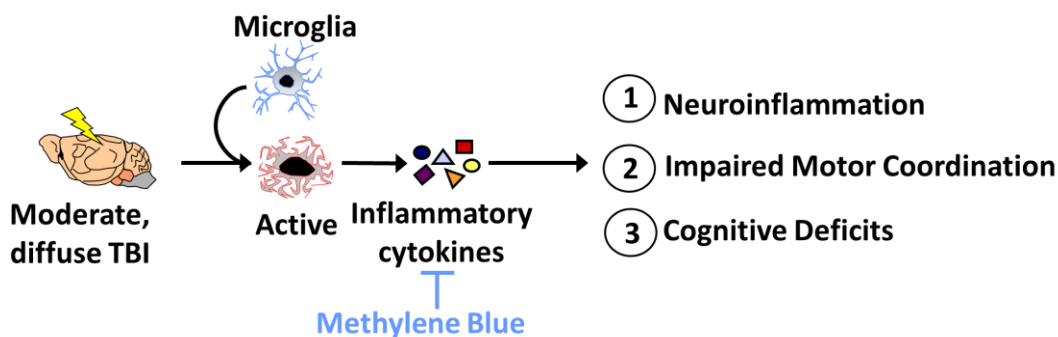


Figure 1. Illustrated TBI effects and methylene blue administration.

Methods:

Animals and Use: Adult (3 mo) male BALB/C mice were used. Mice were housed in polypropylene cages and maintained at 25 °C under a 12 h light/12 h dark cycle with ad libitum access to water and rodent chow in Wiseman Hall. All procedures are in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and have been approved by The OSU Institutional Laboratory Animal Care and Use Committee.

Fluid Percussion Injury (FPI): Mice received a craniectomy 3 mm in diameter mid-way between Bregma and Lambda under 1.5% isoflurane anesthesia. Following recovery from anesthesia (30 min – 1 h), mice received a midline and diffuse, moderate TBI using the Fluid Percussion Injury (FPI) Device (Custom Design & Fabrication by Virginia Commonwealth University, Richmond, VA), which induces injury with a fluid-pulse of saline onto the brain. Sham control mice were subjected to all experimental conditions, excluding the fluid pulse injury (FPI). The FPI model induces a consistent, moderate and diffuse TBI with a 10-20% mortality rate.

Methylene Blue Treatment: Mice received up to three doses of 100 µl i.v. tail-vein injection of vehicle (ddH₂O) or dilute 1% methylene blue solution (Akorn, Inc; 2 mg/kg) 15 min, 12 h, and 24 h following injury. No relevant side effects have been identified in our model or clinically at this dosage.

Lipopolysaccharide: Mice received LPS (0.33 mg/kg BW; serotype 0127:B8, Sigma-Aldrich) intraperitoneally 1 month post injury.

Microglial Isolation: Brains were removed and homogenized 1 month post-injury. Enriched microglia were isolated using a Percoll density gradient extraction protocol [15]. We

have previously determined that this results in an “enriched” population of 85-90% pure microglia. Enriched microglia will then be used for qPCR.

Quantitative PCR (qPCR): mRNA from enriched microglia was isolated using a spin-column PrepEase RNA Spin Kit (Affymetrix); mRNA from HPC was isolated using TRI Reagent® Protocol. mRNA was converted to cDNA and analyzed using qPCR to determine whether pro- (IFN- α , IFN- γ , IL-1, IL-6, and TNF- α) or anti-inflammatory (IL-4R α , IL-4, IL-10, and TGF- β) mediator expression was upregulated, as well as microglial priming (MHCII and CD48) and activation (IL-1 β , TNF α , IL-6, IL-10, IL-4, and CD14) marker expressions as previously described.

Rotarod Assessment: A forced motor activity test served as a measure of functional recovery to evaluate the endurance, balance, and coordination of mice on a rotating cylinder over time. The mice were acclimated to the rotarod three days before testing. On day one, the mice are placed on a stationary rod. On day two, mice began with a constant rod-rotation frequency of 5 rotations per minute (rpm) until complete acclimation for 30 seconds. On day three, mice are familiarized at 10 rpm with 0.2 rpm/s acceleration until failure. On each day of testing, two consecutive trials are given, and then a third trial ten minutes after the second, each at 10 rpm with acceleration at 0.2 rpm/s. If mice fall once before their endurance time on the rod reaches 5 seconds, the trial is restarted. If this occurs immediately a second time, the time is counted. The three trials are done daily for a week after sham/injury.

Barnes Maze: Hippocampal-dependent learning and memory recall were determined using the Barnes maze paradigm as previously described (Bach et al., 1995) with a few modifications. These modifications include using dim light and white noise to serve as an aversive stimulus, to facilitate movement of BALB/c in the maze.

The acclimation phase consisted of 2 trials: (1) mice were guided to the escape hole and allowed 2 min in the escape hole, and (2) with the addition of white noise, mice were guided to an incorrect or “dummy” hole and then guided to the escape hole and allowed 2 min in the escape hole without the aversive stimulus. Trials were initiated 30 min apart for each mouse. Following the acclimation day, the position of the escape box was shifted 180 degrees to its permanent location for the remainder of the paradigm. The acquisition phase consisted of four trials per day for four consecutive days and is used to assess learning. Mice were placed in the center of the maze under a semi-opaque container for 10 s. Recording began when the container was lifted and white noise was simultaneously added. The white noise ceased once the mouse entered the escape hole. Each trial lasted 180 s or until the mouse entered the escape hole. If the mouse did not reach the escape hole in the allotted time then it was guided to the hole. Speed, distance traveled, time to find the escape hole, and the number of primary errors (i.e., errors made before encountering escape hole) were determined. Mice were tracked, recorded, and analyzed using the EthoVision XT 8.5 tracking software (Noldus Information Technology).

Statistical analysis: Data were subjected to the Shapiro-Wilk test using Statistical Analysis Systems (SAS, Cary, NC) statistical software. To determine significant main effects and interactions between main factors, data were analyzed using one-way (i.e., injury and treatment), two-way (i.e. injury×treatment), or three-way (i.e., injury×treatment×time) analysis of variance (ANOVA) using the general linear model procedures of SAS. When appropriate, differences between treatment means were evaluated by an F-protected t-test using the least significant difference procedure of SAS. Edema was evaluated using a one-tailed t-test after ANOVA. Rotarod and Barnes Maze data were further subjected to repeated measures ANOVA. All data are expressed as treatment means±standard error of the mean (SEM).

Results:

Methylene blue attenuated markers of microglial activation 24 hours after injury.

We demonstrate here that two administrations of methylene blue, at 15 min and 12 h post-injury, plays the same role compared to one dose at the 24 h time point. Importantly, two doses of methylene blue, is not toxic to mice. Methylene blue reduced the expression of the inflammatory markers CD14, IL-1 β , and TNF α following a TBI to levels comparable to sham-injured controls (Figure 2).

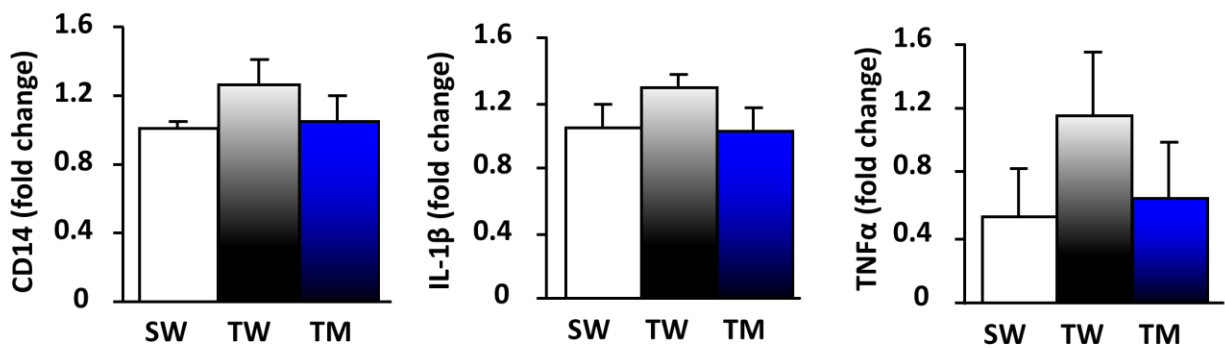
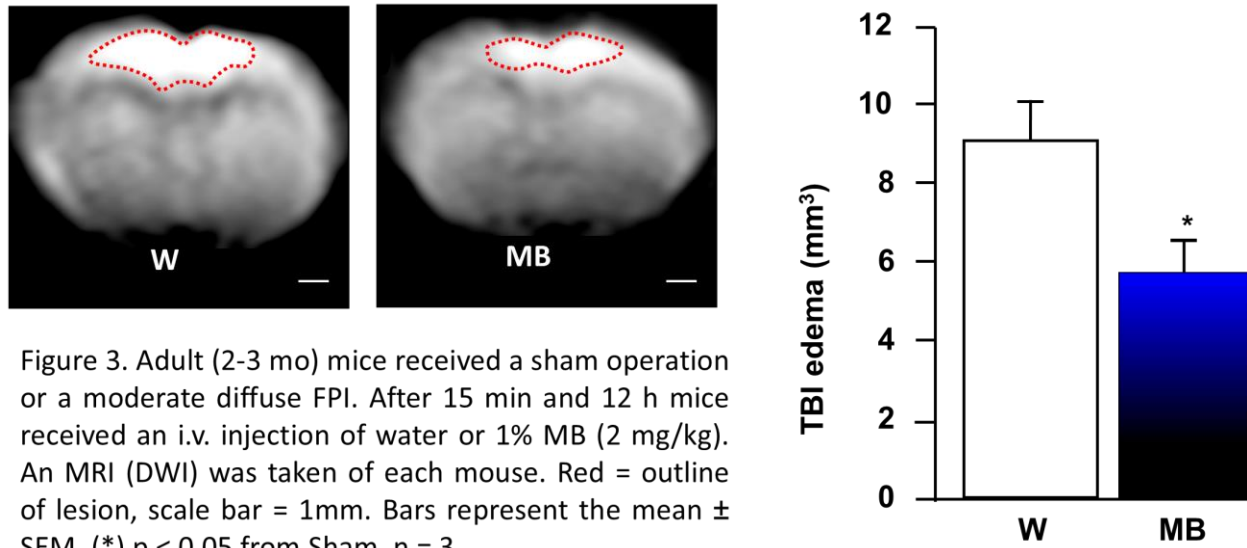


Figure 2. Adult (2-3 mo) mice received a sham operation or a moderate diffuse FPI. After 15 min and 12 h mice received an i.v. injection of water or 1% MB (2 mg/kg). Mice were sacrificed 24 h after injury for hippocampal brain slice and mRNA expression was determined by qPCR. n = 4-6

Methylene blue reduced edema in the brain 24 hours after injury.

Here, two doses of methylene blue, at 15 min and 12 h, reduced edema significantly compared to untreated controls (Figure 3). This was quantified using DWI MRI statistics, and two representative images demonstrate the reduction in edema.



Methylene blue improved motor coordination one week after injury.

Reduction of edema and microglia-associated inflammatory markers have been published with one dose of methylene blue. Using two doses of methylene blue to find the same results was encouraging, but not unique to two doses. As two doses of methylene blue was successful, we increased the treatment to three doses so that methylene blue was at a consistent concentration in the brain over 24 hours. Unlike one dose of methylene blue, three doses of methylene blue improved motor coordination after TBI (Figure 4). Motor coordination was assessed using rotarod, with training beginning 3 days before injury and assessment continuing for seven days after injury. By seven days, mice in all treatment groups reached or exceeded baseline performance. The success of three doses of methylene blue in motor coordination improvement gives promise for success in longer term studies because it suggests that the attenuation of microglial priming and activation plays a larger role in improving overall function (such as motor coordination).

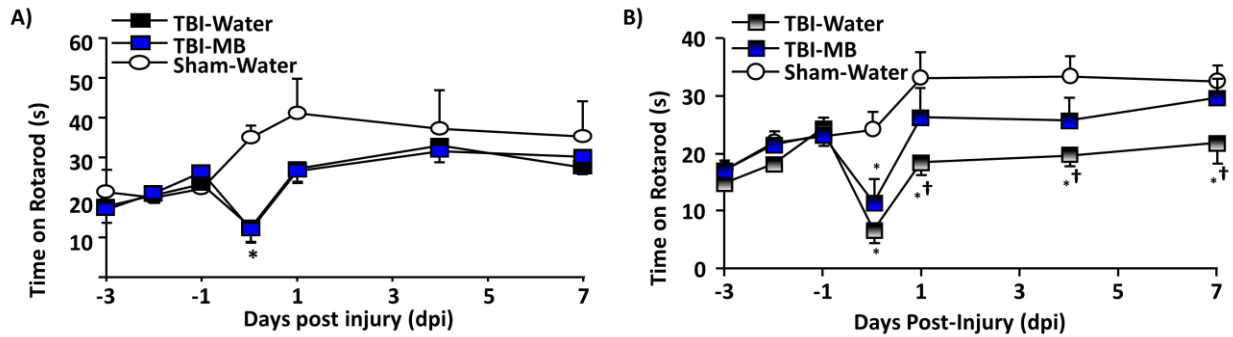


Figure 4. Adult (2-3 mo) mice received a sham operation or a moderate diffuse FPI. After A) 15 min or B) 15 min, 12 h, and 24 h mice received an i.v. injection of water or 1% MB (2 mg/kg). 1-7 d after TBI, mice were tested for motor coordination using the rotarod test and results are shown as time spent on rod. Bars represent the mean \pm SEM. (*) $p < 0.05$ from Sham, (†) $p < 0.10$ from TBI-MB. $n = 8-14$

Methylene blue reduced injury-sensitized, lipopolysaccharide-induced release of inflammatory markers and microglial priming one month after injury.

One month after injury and methylene blue treatment (x3), mice received an immune challenge with lipopolysaccharide (LPS). LPS creates a potent immune response that peaks around 6 h and is alleviated by 24 h in uninjured mice. In mice who have received a traumatic brain injury, the presence of inflammatory markers in the brain and subsequent behavioral deficits persist through 24 h. Here, we observe a trend towards reduction in gene expression of inflammatory markers (IL-1 β , CCL2, CD14, and TNF α) by microglia in the brain 24 h following an immune challenge one month after TBI. This suggests that microglial priming is reduced one month after injury (Figure 3). Thus, three doses of methylene blue has a neuroprotective effect in by alleviating microglial priming and thus the hyperactive release of inflammatory markers one month after treatment.

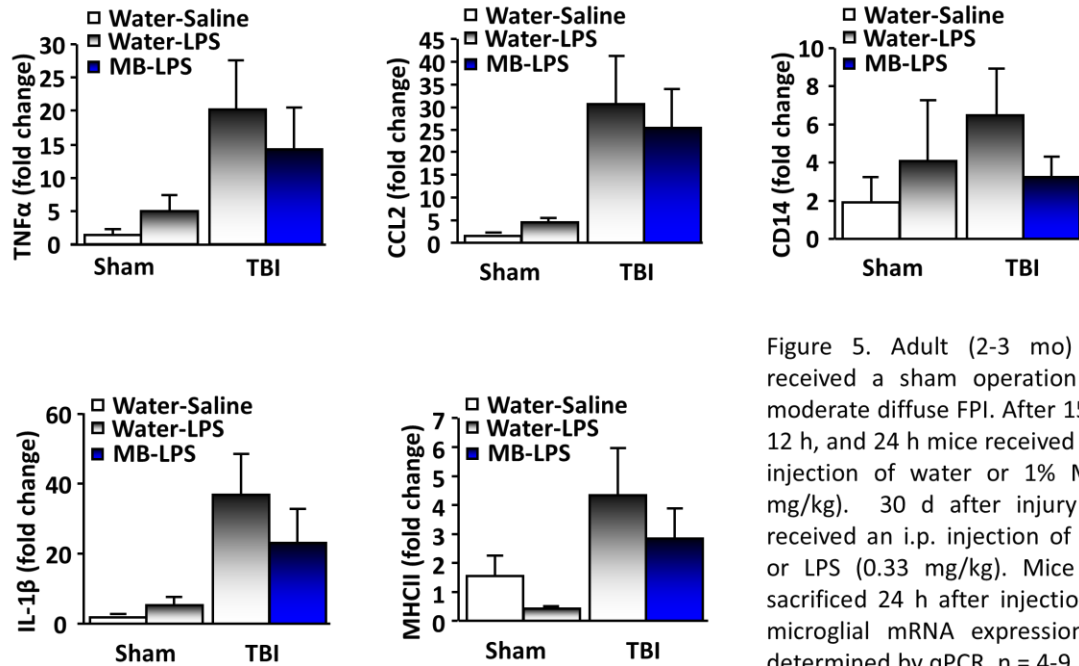


Figure 5. Adult (2-3 mo) mice received a sham operation or a moderate diffuse FPI. After 15 min, 12 h, and 24 h mice received an i.v. injection of water or 1% MB (2 mg/kg). 30 d after injury mice received an i.p. injection of saline or LPS (0.33 mg/kg). Mice were sacrificed 24 h after injection and microglial mRNA expression was determined by qPCR. n = 4-9

Methylene blue attenuated cognitive deficits one month after injury.

Following the observation that methylene blue was able to have longer term effects, such as the blunting of microglial priming and hyper-activation of microglia as observed by reduced inflammatory cytokine release following a peripheral immune challenge (LPS), other more functional processes became of interest. Following a traumatic brain injury, mice suffer from long-term cognitive deficits [20]. Hippocampal-dependent memory and cognition is assessed using Barnes Maze. One month (30 d) following traumatic brain injury and MB, mice were acclimated to the Barnes Maze. Learning was assessed for four consecutive days. Mice that received methylene blue treatment after TBI appeared to trend towards making fewer errors than those that received TBI and vehicle (figure 6). Mice that had received a traumatic brain injury but no treatment made approximately same number of errors throughout the four days of

assessment. Additionally, mice with methylene blue treatment took a time to find the escape hole equal to sham mice by day 3 (33 post injury).

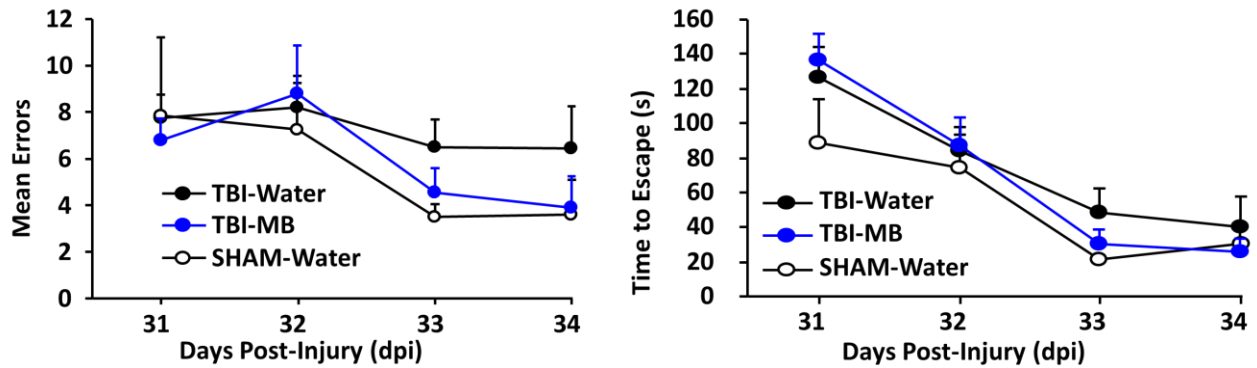


Figure 6. Adult (2-3 mo) mice received a sham operation or a moderate diffuse FPI. After 15 min and 12 h mice received an i.v. injection of water or 1% MB (2 mg/kg). 30 d after injury mice were acclimated to the Barnes Maze. 1-4 d after training, learning was assessed. Bars represent the mean \pm SEM. n = 5-9

Discussion:

We expected these experiments to reveal several novel findings. Specifically, we predicted that multiple doses of MB treatment will attenuate microglial activation and priming, reduce hippocampal expression of pro-inflammatory factors, and ameliorate motor coordination and cognitive deficits following a moderate and diffuse TBI. Furthermore, we expected multiple doses of MB to improve functional recovery compared to vehicle controls. While one dose of MB did not promote improved recovery in motor coordination and nesting behavior, we hypothesized that 2-3 doses of MB will assist mice in their recovery after TBI. As one dose of MB has demonstrated a reduction of pro-inflammatory markers (17), we expected 2 – 3 doses of MB to have a better effect in reduction of brain inflammation after TBI. Before this project, the long-term benefits of MB treatment pertaining to TBI and secondary insult were unknown. Here we show multiple doses of methylene blue reducing inflammatory markers in the brain to sham controls and reducing brain edema by 24 h, and improving motor coordination to sham controls by 7 days. Trends toward reducing microglia hyper-responsiveness to LPS one month after TBI and towards improving hippocampal dependent learning were also observed. This study will aid in the development of treatment strategies for TBI by improving treatment of both acute and prolonged TBI-related deficits. Currently, the lack of an effective TBI treatment is detrimental to society at large. Quick recovery after a TBI will benefit both the individual and society by overcoming the potential for neuropsychiatric complications, reducing hospital/rehabilitation costs, and limiting death.

By limiting microglial activation after injury by using methylene blue, acute complications such as brain edema are attenuated. Edema is found concurrent with neuroinflammation, and there are no current treatment strategies for its treatment [21]. Due to the

craniectomy in the FPI model, it is difficult to contain the edema and thus detect it [22]. Yet, we have previously published that at 24 h TBI-induced edema is present and this is not observed in mice treated with methylene blue [17]. Here we demonstrate that this edema is quantifiable using MRI (DWI). Thus, methylene blue is able to visually reduce edema in the brain 24 h after injury in mice. As MRI is already used in clinical studies to assess TBI severity, this knowledge can be incorporated using often used tools during future methylene blue clinical trials [23].

Motor coordination was assessed via Rotarod, as previously described. We have previously published that by 7 dpi mice have recovered to baseline motor performance [15] but was not influenced by one dose of methylene blue did [17]. Here we show that three doses of methylene blue blunted the reductions in motor coordination observed as soon as one day post injury. While it can now be said that motor coordination is at least partly influenced by anti-inflammatory treatment.

By comparing qPCR-quantified MHCII expression of TBI-MB-LPS mice with TBI-Water-LPS and Sham-Water-LPS mice, we were able to decide the effectiveness of three doses of MB in the context of microglial priming and reactivity. We expected to show TBI-MB-LPS mice to have significantly reduced MHCII levels as compared to mice not treated with MB (TBI-Water-LPS), while having comparable MHCII expression to Sham-Water-LPS mice. This was observed in published studies between TBI-MB mice and TBI-Water mice at 24 h [17]. Additionally, microglia activation was assessed through pro-inflammatory cytokine expression (e.g. IL-1 β). It was expected that, as observed in TBI-associated microglial cytokine secretion at acute time points, pro-inflammatory cytokine levels will be comparable to sham mice 24 h following LPS injection. While these comparison was not observed, a downward trend was

observed in markers of microglial activation and priming with mice treated with methylene blue as compared to mice that received a traumatic brain injury but no methylene blue treatment.

Effectiveness of methylene blue in improving cognition, as assessed by Barnes Maze, was also insignificant. We have previously reported that while mice recover from TBI by 7 dpi, inflammatory profile of microglia remain elevated and persist in the hippocampus 30 dpi. This coincides with deficits in hippocampal-dependent learning [20]. While a similar trend was also observed in mice via number of errors and time to escape, mice appeared statistically indistinguishable.

There are many reasons as to why significance was not determined. Most obviously, statistical analyses were conducted on a cohort of $n = 4 - 9$ mice. While this number of mice tends to reveal significance at closer time points, a greater number of mice appears necessary to extrapolate statistical significance at a later time point. Additionally, dosing may have been inconsistent in three doses due to a number of reasons. Intravenous injection is stressful for mice [24]. Multiple doses of methylene blue used the same constraint and administration system as one dose of methylene blue and mice appeared to remember the traumatic experience by the third time. While the stress that occurs in mice was taken into account, as all mice received three tail-vein injections, the stress was not quantified as its own study. On its own, stress has the ability to activate microglia and thus leads to an increase in pro-inflammatory markers such as pro-inflammatory cytokines and chemokines like IL-1 β and CCL2 [25]. The multiple-injection process is not only stressful, there is also little way to verify that the injections were completed successfully besides the operator's verification. Increased visible animal stress may lead to increased operator discomfort and hesitation, leading to sub-optimal task performance [26]. Usage of a catheter, topical anesthetic on the tail prior to needle placement, and external pump

packs to better quantify injection volume and minimize the restriction of animal movement would all be important to incorporate in future multiple dose experiments in order to better administer methylene blue and account for such unanticipated variables. On the other hand, MB may have an anxiolytic effect in mice at the planned dosage. Three doses of MB, at our concentration and time intervals, leads to a relatively constant level of 2 mg/kg MB for 24 h in the body, mostly concentrated in the brain. This is a much longer duration of concentration than that of our earlier studies with one dose of MB. In fact, MB has been published to have anxiolytic properties in rats [19]. Concentrations of MB doses can be tuned according to results.

As our intended results were not observed, the effectiveness – and perhaps specificity – of three doses of MB as observed with one dose at 24 h may differ from that of a greater dose and further time point. To investigate that three doses of MB is ineffective at alleviating LPS-induced microglial hyperactivity, cell culture experiments with LPS-activated BV2 microglia would take place with three-doses, following the same experimental procedure as published with one dose [17]. If reduced IL-1 β and increased IL-10 messenger ribonucleic acid in microglia are not observed, we can conclude a relatively high dose MB may not directly influence microglial priming and reactivity at 30 d. A secondary approach may then be considered, where co-culture experiments, between microglia and astrocytes can determine whether microglia regulate astrocytes to perform more neuroprotective and reparative tasks when treated with MB [27]. Our data shows that an increased anti-inflammatory and reduced pro-inflammatory profile of microglia stimulates astrocytes, the most abundant neuronal cell, to support intrinsic repair [28]. Finally, in vivo, MB may be affecting endothelial cells and the BBB, and indirectly affecting microglial and astrocytes. In this event, we could use an in vitro model of the BBB see whether this is true [18]. Studies ablating microglia via CSFR1 signaling may also help assess whether

neuroinflammation is due primarily to microglia or microglial priming and hyperinflammation are secondary to an unknown cause [29]. This will help us understand whether microglia are the appropriate cell type to continue efforts related to characterizing methylene blue's efficacy in attenuating brain inflammation and other TBI-related complications.

In the future other aspects of TBI-associated neuropsychiatric complications, such as depressive-like behavior, can be assessed with the forced swim test or sucrose preference [30]. We expect to see a significant decrease in depressive like-behavior post LPS administration in TBI-MB-LPS mice compared to TBI-Water-LPS mice. All mice, as discussed earlier, are expected to recover behaviorally by 7 days post injury. Therefore, TBI-Water-Saline mice will not show any difference in depression at 30 d when compared to previous studies at 7 d post injury. TBI mice that received LPS, on the other hand, are expected to have a hyperactive immune response, prompting elevated depressive-like behavior at 30 dpi. This depressive-like behavior is expected to be attenuated to Sham-Water-LPS levels in MB treated mice, as we have published that MB alleviates TBI depression symptoms to sham levels by 7 days (without LPS injection) [17]. Rotarod can be used following LPS injection to visualize motor coordination following microglial reactivity.

Conclusion:

Our hypothesis is that multiple doses of methylene blue administered immediately after a traumatic brain injury reduces microglial activation and priming following an immune challenge at one month after injury as well as result in the improvement of motor coordination at one week and cognition at one month after injury (figure 1). While the data presented serves as a foundation towards methylene blue's efficacy in the context of TBI-associated complications, more data collection is necessary to fully assert the hypothesis. This study contributes towards the development of treatment strategies for TBI by improving treatment of acute TBI-related deficits and relieving TBI patients from the prolonged recovery and devastating effects of TBI-associated coordination and cognitive deficits.

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